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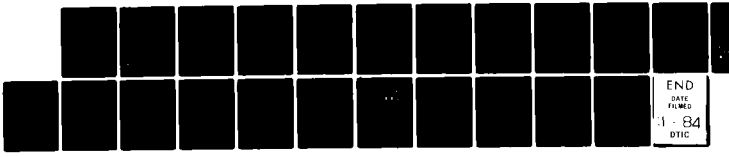

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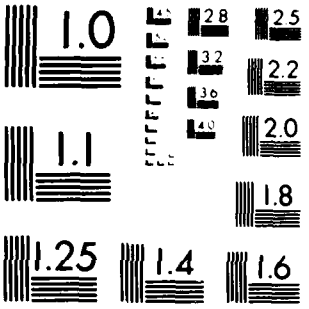

Investigation of Immunoregulatory Alphaglobulin (IRA) in Shock and Trauma

Annual Progress Report

John A. Mannick, M.D.

July, 1981

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The major research accomplishment of the past year included the determination that serum immunosuppressive activity was highly correlated with cutaneous anergy in a series of 21 burn patients with greater than 30% body surface area burn. Anergy in these patients also was significantly correlated with impairment of the response of peripheral blood lymphocytes to PHA stimulation. Low molecular weight peptide containing factions obtained from the serum of burn and trauma patients consistently suppressed the ability of mice to resist challenge with Listeria monocytogenes organisms. Mice injected with this material demonstrated suppressor cell activity in their spleens

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ABSTRACT - Page 2

as evidenced by the effect of their splenocytes on the response of syngeneic spleen cells to mitogens and antigens.

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PROGRESS REPORT

During the past year burn patients have been a major focus of our clinical studies. Burn patients with greater than 30% body surface area burn were skin tested for hypersensitivity responsiveness to 4 standard recall antigens and were sensitized to dinitrochlorobenzene (DNCB). The results of the skin tests were compared on each occasion with the ability of the patient's serum in 10% concentration to suppress normal human lymphocyte stimulation by phytohemagglutinin (PHA) in tissue culture. Seventy-eight serum samples were taken and the immunosuppressive activity related to the presence or absence of coexistant cutaneous anergy (see Figure 1 in the appendix). Cutaneous anergy or relative anergy was present on 40 occasions and 25 (63%) of the associated serum samples at 10% concentration suppressed normal lymphocyte blastogenesis with PHA by more than 50%. There was a normal delayed hypersensitivity reaction on 38 occasions and this was associated with suppressive serum on 11 occasions (28%). By χ^2 analysis there was a correlation between anergy and coexistant serum immunosuppression of greater than 50% ($\chi^2 = 8.71$, $p<0.005$).

The 78 serum samples represented serial measurements in 21 patients. Nine of the 10 (90%) patients that developed anergy also developed suppressive serum at some stage of their illness whereas only 14 of 11 (36%) who did not become anergic developed suppressive serum. The mean greatest serum immunosupression in the anergic group of patients was 65.1% and the mean greatest immunosuppression in the reactive group was 27.7% which is also a significant difference ($p<0.01$ using Student's t test). The relationship of anergy to serum immunosuppression in two patients over the course of their illness is shown in Figure 2 in the appendix. Serum suppressive activity in burn patients did not correlate with plasma levels of PGE_2, as described by radioimmunoassay, with plasma cortisol levels, or levels of circulating endotoxin.

On 33 occasions skin testing and determination of the patient's peripheral blood lymphocyte response to PHA were done synchronously. (see Figure 1 in appendix) On 20 of these occasions anergy or relative anergy was present. A greater than 50% impairment of the peripheral blood lymphocyte PHA response was related to anergy or relative anergy on 15 (75%) of these occasions. On 13 occasions the skin test was reactive and this was associated with lymphocyte suppression in 3 cases (23%). This difference was statistically significant ($\chi^2 = 7.35$, $p<0.01$).

Anergy did not correlate with predicted survival in this population of burn patients but was a good predictor of the actual survival of these individuals. This work has been accepted for publication in the Archives of Surgery (see enclosed reprint)

Most burn patients did not demonstrate an impaired response of their washed peripheral blood lymphocytes to PHA stimulation in vitro. However, a depressed PHA response was associated with severe infection and high mortality. Four of the 7 patients who manifested this finding died. Conversely, only one burn patient died without manifesting severe depression of the peripheral blood lymphocyte response to PHA stimulation in vitro and this patient died of pulmonary embolism. The presence of circulating leukocytes suppressive of the response of lymphocytes from normal donors to PHA was studied serially in 7 burn patients (27 samples) and correlated significantly with depression of PHA-induced blastogenesis ($r=0.72$, $p<0.01$) in these patients. These studies suggest that the presence of a circulating immunosuppressive factor(s) is a very common consequence of major burn injury. Although this circulating immunosuppressive material may inhibit host resistance to infection many patients who manifest this finding survive. However, the development of 50% or more suppression of the response of peripheral blood lymphocytes to PHA as compared with simultaneously studied normal controls and the appearance of circulating suppressor leukocytes is associated with a grave prognosis. This work was mentioned in last year's progress report and has subsequently been published in the Surgical Forum and in the Annals of Surgery (see enclosed reprints).

During the past year we have also begun to study a series of patients who have sustained major accidental trauma (injury severity score (ISS) 20-40) and while these studies are not yet complete they show that major trauma patients resemble burn patients in that suppressive serum is a ubiquitous finding in these individuals and that impending septic death is associated with diminished lymphocyte PHA response.

Investigations of the biological effects of low molecular weight serum suppressive material (Peak 3 or 4 from Sephadex G-25 chromatography) from trauma and burn patients have continued in an animal system. Low molecular weight material from burn patients, from normal individuals and from patients following major trauma has been injected into A/Jax mice which were then challenged with an LD 20 dose of Listeria monocytogenes organisms, usually 1 x 10^5 organisms. Listeria was selected because in common with a number of gram negative bacteria it is known to require an intact cellular immune response for its elimination. Low molecular weight material from burn patients and from trauma patients at a dose of 5 mg per mouse induced 60-100% mortality. Control mice injected with higher molecular weight material from the same burn and trauma serum did not manifest increased mortality and the low molecular weight material administered without Listeria did not induce mortality. We believe that these experiments offer convincing evidence that the circulating low molecular weight suppressive material from burn and trauma patients suppresses host resistance to some microorganisms. This work will be submitted for publication to the Annals of Surgery (see Table I in appendix).

During the past year we have also initiated experiments to determine the effect of low molecular weight material (Sephadex Peak 3 or 4) from trauma and burn patients on the induction of suppressor cells in mice. Preliminary results have shown that the spleens of mice injected with this low molecular weight material are able to suppress the PHA response of normal syngeneic mouse spleen cells in a graded dose response fashion. (see Table II and Figure 3 in the appendix)

During the past year we have also pursued the purification of the active fraction(s) present in immunosuppressive serum from trauma and burn patients. Pools of serum from individuals who have undergone major surgical trauma or have suffered major burns or accidental trauma have been subjected to DEAE cellulose chromatography and the initial two peaks were found to be active. These peaks were further fractionated by gel filtration on Sephadex G-25 columns. The low molecular weight fraction estimated 1000-5000 daltons consistently eluted at 2/3 column volume and was called Peak 3 or 4 depending upon the number of peptide peaks in the sample. This fraction was found to contain a majority of the suppressive activity as determined by its ability to suppress PHA stimulation or normal human lymphocytes and tissue culture without cytotoxicity. We found that the suppressive moiety(s) in the active G-25 peak could not be resolved by ion exchange chromatography or further gel filtration and was of too low molecular weight to be isolated by isoelectric focusing or polyacrylamide gel electrophoresis. Therefore, the active G-25 peak was further fractionated by preparative high-voltage paper electrophoresis at pH 3.5. Individual ninhydrin positive moieties were eluted from the electrophoretogram and recovered by lyophilization. These fractions were tested for suppressive activity in vitro and the majority of the activity appeared to be in a highly basic fraction as noted in the previous progress report. This highly basic molecular species has not been recovered in detectable amounts from similarly processed samples of serum from patients who have suffered minor trauma or from normal volunteers. This basic fraction has also been shown to inhibit antibody formation in the Mishell-Dutton system in vitro and to inhibit the generation of cytotoxic cells in mixed lymphocyte culture (see Figure 4 and Table III in the appendix). It is clear, however, that further work will be necessary before the molecular species responsible for the suppressive activity from peaks 3 or 4 from Sephadex G-25 chromatography can be characterized. We are currently processing more serum pools from trauma and burn patients in an attempt to resolve this problem.

LEGEND

Figure 1: Shows the association of serum suppressive of lymphocyte blastogenesis and impaired blastogenesis of peripheral blood lymphocytes with anergy in 21 burn patients on 78 occasions. It is apparent that suppressive serum and impaired lymphocyte blastogenesis were found much more frequently in anergic patients than in patients with normal delayed hypersensitivity responses.

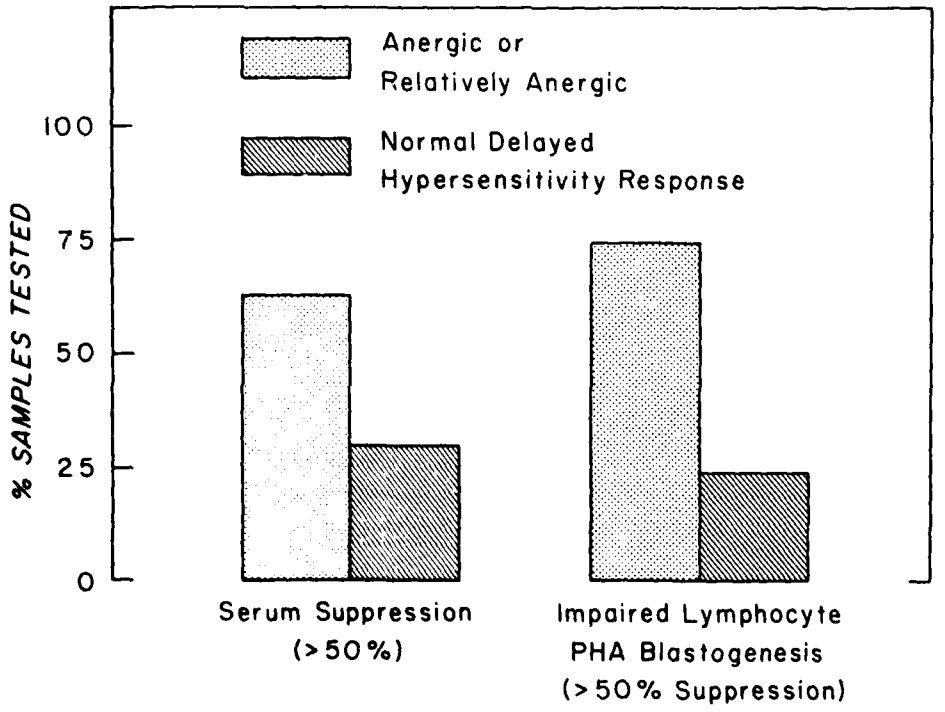

Figure 2: Demonstrated the serum suppressive activity and delayed hypersensitivity responses in two patients over time in the post burn period. Patient I had a 40% burn from which he recovered, patient II had a 30% burn which proved fatal. The persistance of anergy and significantly immunosuppressive serum in the patient with the fatal burn is noteworthy.

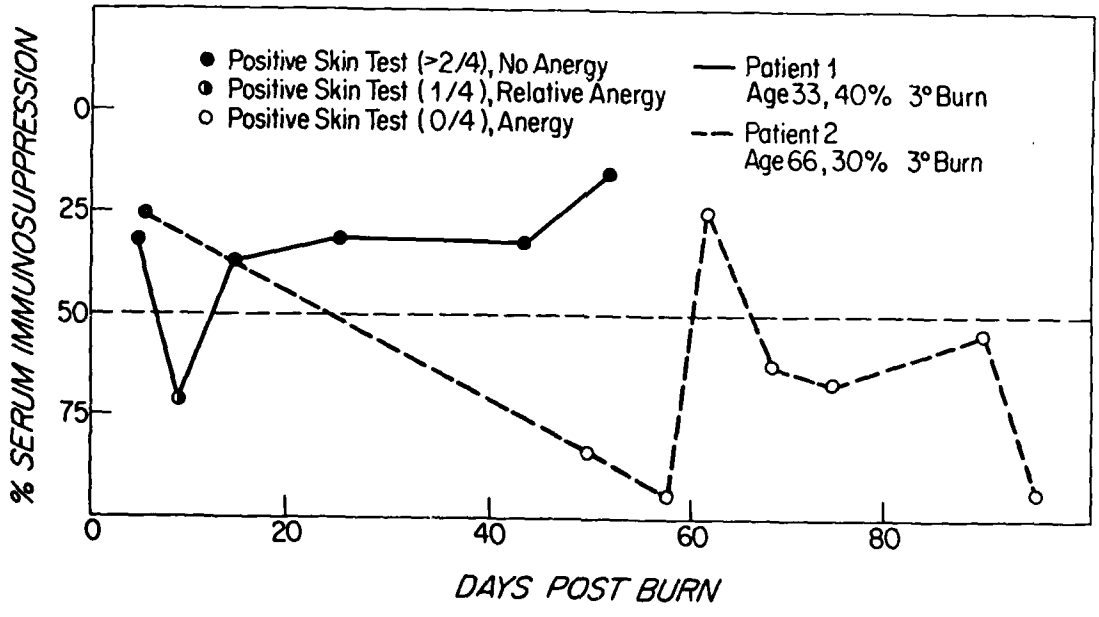

Figure 3 Pooled splenocytes from A/Jax mice that had been injected with 5 mg G-25 Peak 4 from the serum of a burned patient at day 0 were harvested at serial intervals after injection. These splenocytes were than added in graded dosages to 5 x 10^5 normal A/Jax splenocytes which were then exposed to an optimal stimulatory dose of purified PHA. The PHA response, measured as cpm H_3-thymidine incorporation, was compared with the response of 5 x 10^5 normal splenocytes alone. It is apparent that splenocytes from peak 4 injected mice markedly suppressed the response of normal splenocytes to mitogen stimulation. Suppression appeared to be maximal at days 4 and 5 with loss of suppressor activity by day 12 after injection.

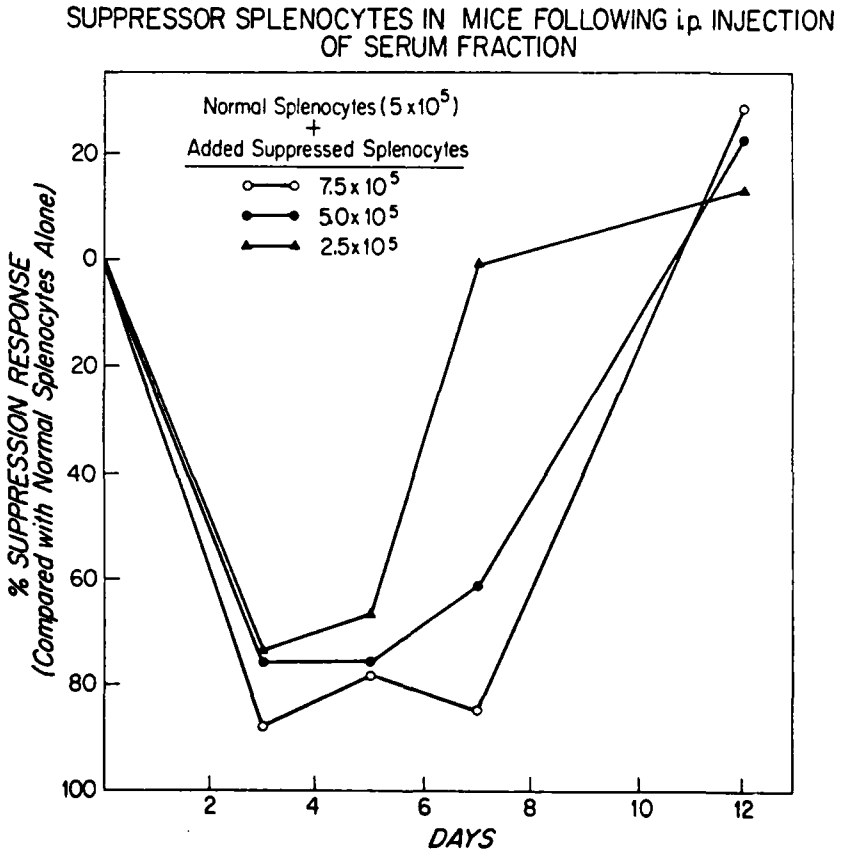

Figure 4 Analytical high-voltage electrophoresis of Sephadex G-25 Peak 4 from pooled trauma patients' serum (PTS), pooled burn patients' serum (PBS) and pooled minor trauma patients' serum (PCTS). Stained with ninhydrin. The ninhydrin positive moiety, slightly more basic than the lysine marker (K), found in trauma patients' serum and burn patients' serum but not in the serum from minor trauma patients or from normal volunteers contained the majority of the immunosuppressive activity when recovered by preparative high-voltage electrophoresis in one or two fraction cuts and tested for its ability to inhibit PHA-induced blastogenesis of normal human peripheral blood lymphocytes (see McLoughlin et al in Appendix), to inhibit the generation of the cytolyti· ells in mouse mixed lymphocyte cultures (see Table I in Appendix), and to inhibi ..e plaque-forming cell response to SRBC in the Mishell-Dutton system (see T in Appendix).

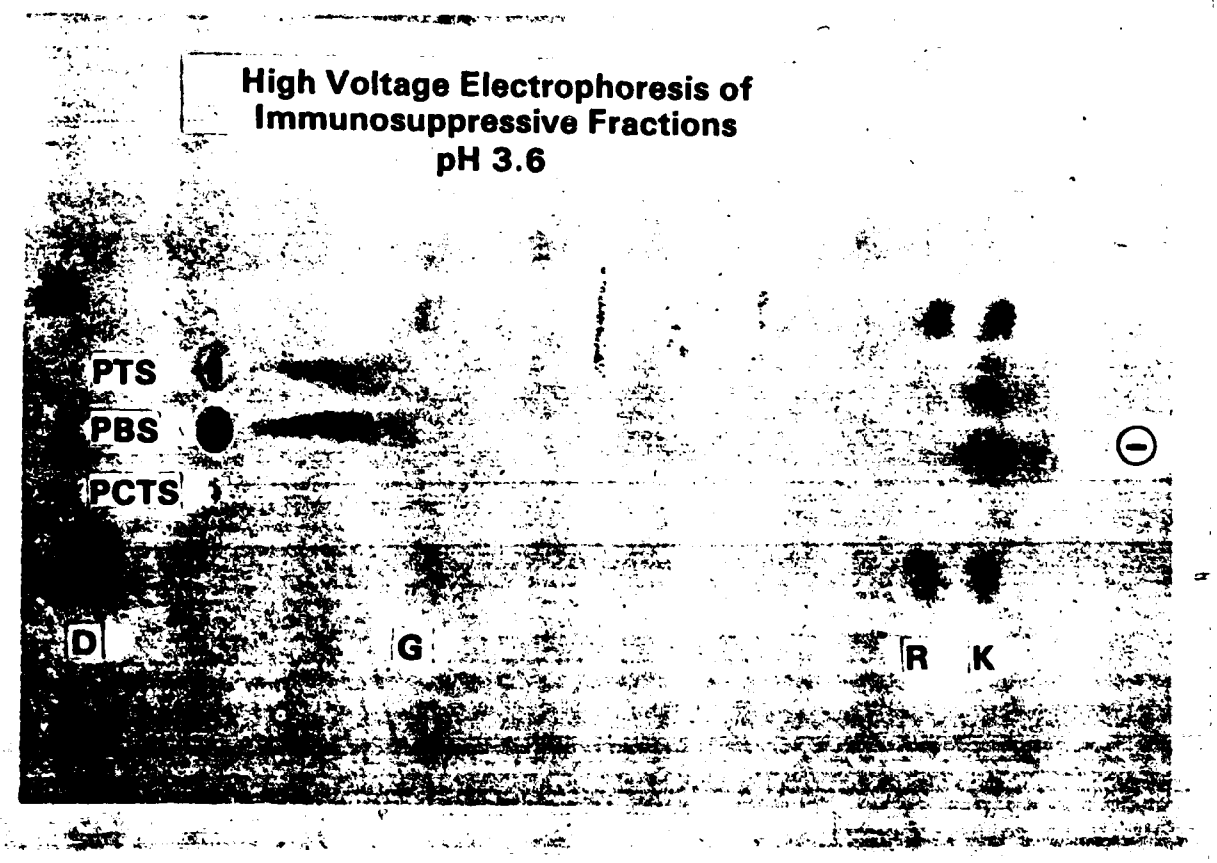

Table I

Effect of Serum Fractions

Source of Serum	% Suppression Normal Human Lymphocyte PHA Response		Mortality from *Listeria* infection in mice (% increase over control)	
	Peak 1	Peak 4	Peak 1	Peak 4
Aneurysmectomy Pt.	15	100	0	40
Aneurysmectomy Pt.	31	50	40	80
Burn Pt.	38	97	0	80
Pooled Burn	*+5	82	0	30
Pooled Trauma			0	40
Pooled Normal	+2	+24	0	0

Peaks 1 and 4 from G-25 Sephadex chromatography of patient serum were tested at 1 mg/ml for suppression of the PHA response of lymphocytes from normal donors. 5 mg of the fractions were injected i.p. into A/Jax mice which were then challenged 24 hr. later with 1×10^5 *Listeria monocytogenes* organisms.

* + = stimulation

TABLE II

Induction of Suppressor Cells
in Mice Injected with Patient Serum Fractions

No. and Source of Mouse Splenocytes	% Suppression** of Splenocyte PHA Response by Trauma Serum Fractions	
	Peak 1	Peak 4
1×10^6 injected*	49	92
5×10^5 normal + 7.5×10^5 injected	23	92
5×10^5 normal + 5×10^5 injected	18	93
5×10^5 normal + 2.5×10^5 injected	14	83

	by Burn Serum Fractions	
	Peak 1	Peak 4
1×10^6 injected	14	71
5×10^5 normal + 7.5×10^5 injected	+7***	88
5×10^5 normal + 5×10^5 injected	+33	53
5×10^5 normal + 2.5×10^5 injected	+10	24

*splenocytes from A/Jax mice injected six days previously with 5 mg. of peaks 1 or 4 from G-25 Sephadex chromatography of trauma or burn patient serum.

**Compared with 5×10^5 normal mouse splenocytes.

*** + = stimulation.

TABLE III

Effect of Fractions Eluted from High Voltage Electrophoretograms of Pooled Major and Minor Trauma Patient Serum (Sephadex G-25 Peak 4)

Fraction No.	% Suppression Mishell-Dutton Assay 0.1 ml.*	0.025 ml.	% Target Cell Lysis after MLC (concentration of fraction approx. 0.1 ug/ml)	
Pooled Major Trauma Serum				
+ 1	0	0		
2	21	0		
3	8	4		
4	27	7		
5	16	3	24.4	
6 **	47	22	2.7	$p < 0.01$
7 **	50	26	11.0	$p < 0.01$
- 8	21	2		
paper blank	9	0	30.5	
Pooled Minor Trauma Serum				
+ 1	26	0		
2	0	0		
3	13	0		
4	19	0		
5	9	9		
6	5	0		
7 **	21	0	34.6	
8 **	17	0	30.0	
9	17	0	43.0	
- 10	3	0		
paper blank	8	0	45.4	

** basic area near lysine marker - see fig. 1
* 0.1 ml. of approx. 1 ug/ml solution of fraction

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Correlation Between Anergy and a Circulating Immunosuppressive Factor Following Major Surgical Trauma

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In order to clarify the relationship between anergy and immunosuppressive activity in the serum, we studied 46 previously well patients before and at three, five, seven and 28 days after surgery. Delayed hypersensitivity was measured by skin testing with four common recall antigens, and serum immunosuppressive activity was determined by the ability of the patient's serum in 10% concentration to suppress by 50% or more the phytohemagglutinin (PHA) stimulation of normal human lymphocytes as compared to pooled normal serum. Prior to surgery, all patients manifested delayed hypersensitivity to one or more antigens, and no patient had immunosuppressive serum. Fifteen patients underwent minor surgery under general anesthesia and did not develop anergy or immunosuppressive serum. Thirty-one patients underwent major cardiovascular surgery. Thirteen of these patients became anergic by day 3 after operation, and 11 of the 13 developed immunosuppressive serum. Eighteen patients maintained delayed hypersensitivity after major surgery, and only three developed immunosuppressive serum. The correlation between anergy and immunosuppressive serum was highly significant ($p < 0.001$). There was a significant difference in the degree of suppressive activity in the serum of the anergic and reactive patient groups for each postoperative day studied until day 28, when there was recovery of delayed hypersensitivity and lack of immunosuppressive serum. The occurrence of postoperative anergy and immunosuppressive serum was not related to the patient's age, sex, number of perioperative blood transfusions or duration of anesthesia but was associated with an increase in postoperative infectious complications ($p < 0.05$) and in postoperative days in the hospital ($p < 0.01$). Pooled immunosuppressive serum from anergic patients was fractionated by ion exchange chromatography, gel filtration and preparative high voltage electrophoresis. The majority of the immunosuppressive activity could be accounted for by an electrophoretically homogenous polypeptide-containing fraction not identified in the serum of patients undergoing minor surgery or in normal individuals. We conclude that anergy occurring after major operative trauma is associated with the appearance of a circulating immunosuppressive molecular species and that these events are in turn associated with increased patient morbidity and increased length of hospitalization.

ANERGY IS FOUND in surgical patients with nutritional deprivation or advanced malignancy and is associated with an increased incidence of sepsis and mortality.[3-6,8] Since restoration of delayed hypersensitivity responsiveness has been reported in such patients following parenteral hyperalimentation,[3,9] this suggests an underlying mechanism for the anergy observed in these depleted individuals. On the other hand, there are conflicting reports as to whether or not major surgical trauma in nondepleted patients is followed by anergy.[8,10] Moreover, if anergy does occur under these circumstances, it is not clear what the mechanism is and what effect, if any, the anergic state has on patient morbidity and mortality. A good deal of recent investigative work has focused on defects in polymorphonuclear leukocyte function detected in anergic surgical patients.[1,4,6] While polymorphonuclear leukocytes clearly play an important role in the defense against bacterial infection in man, they have not been shown to be obligatory participants in delayed hypersensitivity responses.[11] These responses are mediated by specifically sensitized T lymphocytes, which in turn elicit the nonspecific cooperation of macrophages.

We have recently reported that major operative trauma is often followed by the appearance in the serum of a circulating factor or factors suppressive of T-lymphocyte activation.[2] We therefore undertook the present investigation in a group of well-nourished

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TABLE 1. *Skin Test: Delayed Hypersensitivity Response to Four Recall Antigens (Mumps, SK-SD, PPD, Candida)*

	Grade	Response (Diameter of Induration)
Anergic	0	0
	1	< 5 mm for 1
Reactive	2	≥5 mm for 1 or ≥3 mm for 2
	3	≥10 mm for 1 or ≥10 mm for 1 and ≥5 mm for another
	4	≥20 mm for 1 or ≥15 for 1 with 10 mm for another or ≥10 mm for 2 or more

surgical patients, none of whom had malignancy, to determine whether or not the appearance of circulating immunosuppressive factors in the serum postoperatively was associated with the manifestation of anergy and whether the anergic state was in turn associated with an altered patient prognosis. We were also concerned with the purification and characterization of the immunosuppressive substance or substances detected in the serum of these surgically traumatized patients.

Patient Population and Methods

Forty-six patients were studied. Fifteen of these patients received general anesthesia for minor surgical operations. Seven underwent inguinal hernia repair, two had dilatation and curettage, three had multiple dental extractions, and three had orthopedic manipulations. The age of this patient population ranged from 35 to 75 years, with a mean age of 56 years. There were 13 males and two females.

Thirty-one patients underwent major cardiovascular surgery under general anesthesia. Sixteen of these patients had abdominal aortic aneurysm resections. Ten underwent coronary artery bypass grafts, and five underwent aortic or mitral valve replacement, with or without coronary artery bypass grafting. The age of this patient group ranged from 42 to 75 years. The mean age was 62 years. There were 27 males and four females. No patient judged clinically to be nutritionally depleted was included in this study, and no patient had cancer. In addition, nine healthy normal volunteers, ranging in age from 24 to 63 years, were used as control serum donors in some of the studies. Informed consent was obtained from all patients before studies were initiated.

All patients were skin tested with four recall antigens for delayed hypersensitivity responsiveness 2 days prior to surgery. The antigens were mumps skin test antigen (Eli Lilly & Co., Indianapolis, IN), 0.1 ml; 50 units of streptokinase-streptodornase (SK-SD) (Lederle Laboratories, Pearl River, NY); intermediate strength tuberculin purified protein derivative (PPD) (Merck, Sharp and Dohme, West Point, PA), 0.1 ml; and *Candida* skin test antigen (Greer Laboratories, New York, NY), 0.1 ml. All skin tests were read at 24 and 48 hours and were scored according to the system listed in Table 1. Responses were graded by the diameter of the area of induration. Patients were considered to be anergic if they had responses of grades 0 and 1 and reactive if they had responses of grades 2, 3 and 4. No patient who was anergic preoperatively was included in these investigations. Skin tests were repeated in all patients on the second postoperative day, the seventh postoperative day and the twenty-eighth postoperative day.

Histamine (Eli Lilly), 0.5 mg, was injected intradermally as a control for an intact inflammatory response in the postoperative period. Thirty-milliliter venous blood samples were drawn beginning 2 days preoperatively and then on the third postoperative day, the fifth postoperative day, the seventh postoperative day, the fourteenth postoperative day (in some patients) and on the twenty-eighth postoperative day. In order to obtain serum samples, the blood was allowed to clot and retract, and the serum was removed by centrifugation at 2000 × g for 30 min and stored in the cold.

In Vitro *Assay of Immunosuppressive Activity*

Serum samples were tested for immunosuppressive activity *in vitro* by studying their ability to inhibit phytohemagglutinin (PHA)-induced normal human lymphocyte proliferation. Heparinized venous blood was obtained from the normal donors, and after gravity sedimentation of the erythrocytes for 2 hours at 20°, the serum layer was placed on sterile nylon wool columns and eluted with Eagle's minimal essential medium (MEM). After washing in MEM, the cells were counted and tested for viability by trypan blue dye exclusion. The procedure yielded a preparation of small lymphocytes, 95% or more pure and 95% or more viable. The micro method was used for testing lymphocyte stimulation. In the wells of Microtest[R] plates (Falcon Plastics) 2.5×10^5 lymphocytes were placed in 0.2 ml of MEM containing 1% glutamine, 5% fetal calf serum, 100 units of penicillin and 100 μg of streptomycin per milliliter and a range of stimulatory doses of purified PHA (2.5, 5 and 10 μg/ml). Serums to be tested for immunosuppressive activity were added to the culture medium in 10% concentration. Controls included cultures with no additions and those with 10% pooled normal serum. The same normal serum pool was used for all experiments. Microtest plates were then in-

cubated in a 5% CO_2 water-saturated environment at 37° for 48 hours. ^{3}H-thymidine, 1 μCi, was then added to each well. The cultures were processed 16–18 hours later by a Mash II® microharvester (Microbiological Associates) and counted in a Packard liquid scintillation counter. All determinations were performed in triplicate. Some wells in each experiment were used for a trypan blue viability determination of the cells incubated with or without the serums being tested. No cytotoxic serums were found in these experiments. Immunosuppression *in vitro* was calculated by the formula

$$\% \text{ Suppression} = 1 - \frac{\text{CPM, experimental wells with PHA} - \text{CPM of control wells without PHA}}{\text{CPM of control wells with PHA} - \text{CPM of control wells without PHA}} \times 100$$

In these studies, suppression of PHA stimulation by experimental serum of 50% or more when compared with control serum was considered significant.

Isolation of Suppressive Material from the Serum

Ten-milliliter samples of serum were fractionated by diethylaminoethyl (DEAE) cellulose ion exchange chromatography in 0.005 M acetate buffer (pH 5.0). The protein peaks from this separation were then recovered by lyophilization and tested for immunosuppressive activity in tissue culture as described above. Lyophilized protein, 100 mg, was then dissolved in distilled water and placed on a G-25 Sephadex® column. The protein peaks from this column were then similarly recovered by lyophilization. After testing for immunosuppressive activity *in vitro*, the G-25 fractions were dissolved in distilled water and acetic acid, pH 3.5, and placed in 10–20-mg aliquots on paper strips for high voltage electrophoresis along with reference amino acids. A portion of the electrophoresis strip was stained with ninhydrin, and the remainder of the strip was cut into fractions containing the various ninhydrin staining moieties. The fractions were then eluted from the paper with distilled water-acetic acid solution and recovered by lyophilization. Each of the recovered, presumably peptide-containing moieties was then tested for suppressive activity in tissue culture.

In Vivo *Assay of Immunosuppressive Activity*

To confirm the results of the *in vitro* tissue culture assay, suppressive and nonsuppressive fractions obtained by G-25 gel filtration were tested for immunosuppressive activity *in vivo* in mice by the Jerne hemolytic plaque assay. Adult C3H mice (Jackson Laboratories) were injected intraperitoneally with test or control fractions 24 hours before the intraperitoneal injection of 4×10^8 sheep erythrocytes. Four days later their spleens were harvested, and the numbers of plaque-forming cells were enumerated as described previously.[7] Duplicate determinations were performed in each of five animals per experimental group.

Cortisol Determinations

Serum cortisol determinations were performed in the hospital laboratory by the competitive protein-binding method.

Results

Correlation of Anergy with Suppressive Activity in the Serum

Of the 15 patients undergoing minor surgical procedures under general anesthesia, no patient became anergic at any time in the postoperative period and no patient developed immunosuppressive serum as determined by the ability of the serum in 10% concentration to suppress by 50% or more the PHA stimulation of normal human lymphocytes (Table 2). However, of the 31 patients who underwent major cardiovascular surgery, 13 became anergic to the skin test antigens by postoperative day 3, and of these 13 patients, 11 developed immunosuppressive serum. Of the 18 patients who remained reactive to the skin test antigens, only three developed immunosuppressive serum at any time in the postoperative period. By χ^2 analysis the correlation between immunosuppressive serum and anergy in the patients undergoing major cardiovascular surgery is highly significant ($p < 0.001$).

As shown in Figure 1, the suppressive activity in the serum of the entire group of patients who became anergic in the postoperative period was significantly

TABLE 2. *Correlation of Anergy with Suppressive Serum Three Days After Surgical Trauma*

Surgery	Skin Test	Immunosuppressive Serum*	Nonsuppressive Serum	Total No.
Minor	Positive	0	15	15
	Negative	0	0	0
Major	Positive	3	15	18
	Negative	11	2	13

* Immunosuppressive serum at 10% concentration was more than 50% suppressive of PHA stimulation of normal human lymphocytes.
† Chi square with Yate's correction: $^*p < 0.001$.

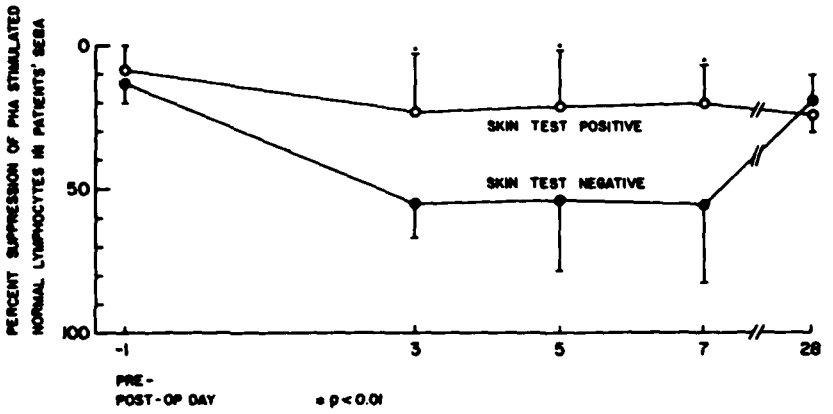

FIG. 1. The immunosuppressive activity in the serum of negative skin test and positive skin test patients following major cardiovascular surgery in the present study. Results are presented as mean percentage suppression of PHA stimulation of normal peripheral blood lymphocytes (± S.D.) by test serum in 10% concentration. The two groups differ significantly on postoperative days 3, 5 and 7 as determined by the paired *t*-test ($p < 0.001$).

greater than that in the serum of patients who remained reactive to the skin test antigens for the first 7 days postoperatively. By day 28 when the patients were seen as outpatients, significant suppressive activity in the serum had disappeared. By day 28 the patients had also regained responsiveness to the skin test antigens. All patients responded normally to intradermal histamine in the early postoperative period. Serum cortisol levels were within the normal range in pooled and selected individual postoperative samples.

Association of Anergy and Immunosuppressive Serum with Patient Morbidity

As noted in Table 3, 11 of the patients undergoing major surgery developed both anergy and immunosuppressive serum in the postoperative period, while 15 manifested neither. The two groups did not differ significantly from one another in age or sex. They also did not differ from one another with respect to the number of perioperative blood transfusions received or the duration of anesthesia. However, the patients with anergy and suppressive serum developed significantly more infectious complications that required antibiotic therapy in the postoperative period. These were predominantly pulmonary and urinary tract infections. Also the group of patients with anergy and suppressive serum spent significantly more days in the hospital postoperatively.

Isolation of Suppressive Material from the Serum

Pooled immunosuppressive serum from eight of the patients undergoing major surgery who developed anergy and suppressive serum in the postoperative period was subjected to DEAE cellulose ion exchange chromatography. The peaks obtained were tested for immunosuppressive activity at 5, 2 and 1 mg/ml. Controls included pooled serum from eight patients who underwent minor surgical procedures and pooled serum from eight normal individuals. Suppressive activity from the immunosuppressive serum from patients in the major surgical group was found principally in the first two peaks from the DEAE column.

These suppressive peaks from DEAE chromatography were then subjected to gel filtration on a G-25

TABLE 3. *Major Surgical Patients*

Anergy and Suppressive Serum	Blood Transfusions	Duration of Anesthesia (hr)	Postop. Infectious Complications	Postop. Days in Hospital
Yes n = 11, M = 10, F = 1, av. age = 63	2.33 ± 0.89*	4.02 ± 0.31*	6/11 $p < 0.05$	13.5 ± 4.2* $p < 0.01$
No n = 15, M = 13, F = 2, av. age = 62	2.33 ± 0.87	4.11 ± 0.24	1/15	8.8 ± 2.2

* Mean ± S.D.

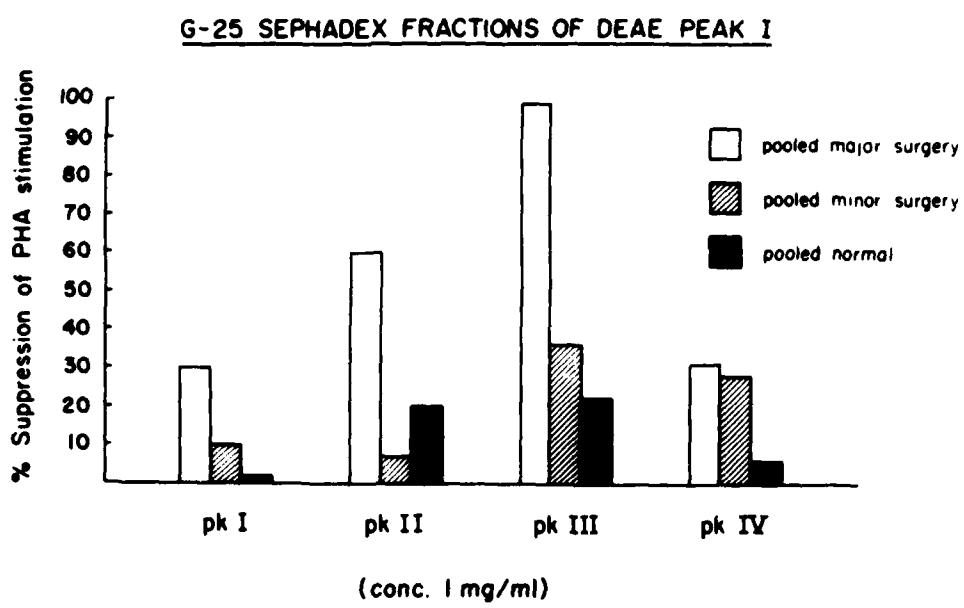

FIG. 2. Suppressive activity of fractions of serum obtained by G-25 Sephadex chromatography and tested at 1 mg/ml concentration. Results are presented as mean percentage suppression of PHA stimulation of normal human lymphocytes. It is apparent that the suppressive activity in the serum of patients following major cardiovascular surgery is found chiefly in peak 3. Pooled serum from patients undergoing minor surgery or from normal individuals contains little suppressive activity.

Sephadex column, and the resultant polypeptide peaks were lyophilized and tested for immunosuppressive activity in tissue culture at concentrations of 2, 1 and 0.5 mg/ml. As noted in Figure 2, the immunosuppressive activity in the serum from suppressed major surgical patients was located principally in peak 3 from the G-25 column. Very little suppressive activity was recovered from serum from patients subjected to minor surgery or from normal individuals.

G-25 peak 3 from suppressed patients undergoing major surgery was also tested for immunosuppressive activity in the mouse. It is apparent from Table 4 that G-25 peak 3 at a dose of 5 mg per mouse produced very significant suppression of the plaque-forming cell response to sheep erythrocytes in contrast to G-25 peak 1. At this dosage the concentration of G-25 peak 3 in mouse serum was calculated to be approximately the same as in the serum of suppressed major surgical patients.

TABLE 4. *Effect of Major Surgery Serum Fractions on Direct Plaque-Forming Cell Response to SRBC in C3H Mice*

Treatment	Plaques per 10^6 Spleen Cells (±S.D.)	Per Cent Suppression
None	485 ± 7.7	
G-25 peak 1		
5 mg	502 ± 22.4	0
1 mg	464 ± 6.1	4
G-25 peak 3		
5 mg	212 ± 12.1	57
1 mg	466 ± 15.6	4

Finally, G-25 peak 3 from suppressed patients undergoing major surgery was further fractionated by preparative high voltage electrophoresis. It was found that the immunosuppressive activity was recovered principally in a highly basic fraction, fraction 11 (Table 5). Fraction 11 was not detectable in the serum of patients who had undergone minor surgery or in the serum of normal individuals. The same area in the electrophoresis strip contained negligible immunosuppressive activity in these control groups.

Fraction 11 from suppressed major surgical patients was subjected to acid hydrolysis and yielded amino acids plus a so far unidentified ninhydrin staining basic component, more basic than known amino acids but less basic than known polyamines.

Discussion

These results clearly demonstrate that following major cardiovascular surgery, temporary anergy fre-

TABLE 5. *Serum Fractionation: High Voltage Electrophoresis of G-25 Sephadex Peak 3 (% Suppression of PHA Stimulation at 1–2 μg/ml)*

		Fraction											
	⊕	1	2	3	4	5	6	7	8	9	10	11	⊖
Major surgery		14	12		24		17	21	0	24		63	
Minor surgery		0	13	0		5	12	11	13	3	0	10	
Normal		0	0	9	0	5	35	13	7	8	37	0	

quently appears in apparently well-nourished patients who do not have malignancy. Our results do not agree entirely with those reported by Slade et al.,[10] who found that patients undergoing major surgery consistently show decreased delayed hypersensitivity responses postoperatively, and our findings are also clearly at variance with those of Pietsch et al.[9] who concluded that skin test responses were not altered by major surgery in apparently nondepleted patients. The reasons for these discrepancies are not entirely clear, but the magnitude of the operative trauma may well be related to the incidence of postoperative anergy in a well-nourished patient population.

Anergy in the patients in the present study was accompanied by the appearance of circulating immunosuppressive activity in the serum, which blocked the activation of T lymphocytes from normal individuals but was not cytotoxic to these cells. The appearance of anergy and immunosuppressive activity in the serum was not the direct result of general anesthesia, since a group of 15 patients undergoing minor surgery under general anesthesia developed neither anergy nor immunosuppressive serum in the postoperative period. Among the patients undergoing major cardiovascular surgery, those who developed anergy and immunosuppressive serum could not be distinguished from those who did not on the basis of age, sex, number of perioperative blood transfusions or duration of anesthesia. However, patients with anergy and immunosuppressive serum had significantly more infectious complications requiring antibiotic therapy in the postoperative period than those patients who remained responsive to recall antigens and did not develop immunosuppressive serum. While there were no postoperative deaths, patients with anergy and immunosuppressive serum also spent significantly more days in the hospital postoperatively than patients who remained responsive.

The mechanism underlying the appearance of anergy and immunosuppressive serum in some of the patients undergoing major cardiovascular surgery in the present study remains obscure. Anergy in these patients cannot be explained on the basis of a generalized incapacity to mount an inflammatory response, since all patients responded normally to intradermal histamine. None of the patients was apparently nutritionally depleted, and no patient had a known malignancy. We have previously demonstrated that immunosuppressive serum in surgical patients is not related to serum cortisol concentrations,[2] and serum cortisol determinations in the patients reported here showed normal levels postoperatively. The most obvious explanation for the present observations appears to be that major operative trauma in itself triggers a temporary inhibition of cellular immunity, possibly mediated by circulating immunosuppressive factors.

The present results also shed some light on the nature of the immunosuppressive activity in the serum of the patients who became anergic after major surgery. In these individuals the majority but not all of the immunosuppressive activity can be accounted for by a polypeptide-like fraction which from its behavior on gel filtration has a molecular weight of approximately 1000 daltons. This material can be recovered as a homogeneous molecular species by high voltage electrophoresis. The highly basic character of this material on electrophoresis makes it unlikely that it is a conventional polypeptide; however, it is not as basic as any of the known polyamines. This material was not recoverable in detectable quantities by identical fractionation of the serum from normal individuals or from patients who had undergone minor surgical procedures.

It is tempting to speculate that the appearance of an immunosuppressive molecular species in the serum of patients following major surgical trauma may be the cause of the anergy seen in these individuals, since there is a statistically significant association between these phenomena. The fact that the gel filtration fraction in which the patients' serum suppressive activity was concentrated also suppressed the ability of mice to mount an antibody response to a T-cell dependent antigen adds weight to this hypothesis. However, a causal relationship between this suppressive substance and clinical anergy cannot yet be claimed to be established.

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Discussion

Dr. George H. A. Clowes, Jr. (Boston, Massachusetts): Specifically in relation to John Mannick's paper, the more I look at trauma and sepsis, the more I realize that they have the same effects metabolically, and in a variety of other ways physiologically. That is, if we assume that the trauma patient has gone beyond the shock phase. The response is very similar then to that of the septic patient.

For some time we have been interested in circulating factors of the small nonprotein peptide type that Dr. Mannick has discussed. (slide) This is a thin-layer chromatograph with a ninhydrin stain that shows in that fraction around 3000–5000 molecular weight a great difference between normal plasma and septic plasma.

Here is what is found when that same fraction from 1000 to 10,000 molecular weight is examined by column chromatography. The point is that the 206 nanometer light exposure, which activates the peptide bonds, demonstrates two large peaks in the fraction from a septic or traumatized patient that are just barely discernible in the plasma fraction from a normal person. This demonstrates that there are many peptide substances circulating under these conditions that are not present in the normal individual.

(slide) It is possible to show a remarkable correlation of the presence of these substances with the clinical state. If one binds these peptides to Sephadex, it is possible to make an antibody. We see here reactions in a series of septic patients, which are absent in the normal person when this immunologic diffusion test is done.

(slide) What is the significance of this? This slide emphasizes the importance of how these agents affect the metabolism of incubated muscle cells. We see virtually no change in the metabolism when saline solution is added. There is a response to insulin in terms of CO_2 production. If normal human plasma or normal plasma fraction are added we get the same response, but if we add the septic plasma, or the septic fraction, we get a suppression of insulin response. That is just one metabolic phenomenon. I can tell you from experience that the same thing happens for protein synthesis and a variety of other parameters which we have measured.

What I'm really saying is that, to me, the bottom line of all this work, and the important response in which we are interested is protein synthesis. After all, T-cell function depends on its ability to make a protein pretty quickly, and I would say that this probably is the common denominator in all of these reactions. The same agent that Dr. Mannick has so elegantly demonstrated to you this morning as well as many other peptides are probably at work in the other phenomena the other two speakers described this morning.

Dr. Jonathan L. Meakins (Montreal, Quebec): Dr. Mannick's paper is a very exciting one, and approaches the problems of immune regulation in a normal, well-nourished population. We have approached this from a slightly different point of view, in terms of decreased host resistance to infection and immunoregulation.

(slide) Dr. Christou recently presented this information on the effect of anergic patients' serum on neutrophil and lymphocyte chemotaxis. The test cells are normal, and it is apparent that relatively anergic and anergic serum reduces PMN chemotaxis and lymphocyte chemotaxis to the anergic range.

(slide) Trauma patients, studied in the emergency department as they are admitted to the hospital, are seen to have abnormal chemotaxis 2–12 hours after injury. Anergy subsequently developed in all of them. This abnormality of their chemotaxis must surely be mediated by a serum factor to appear so promptly.

(slide) We have looked at the concentration of anergic serums required to inhibit chemotaxis and find that there are two inflection points of inhibition of PMN chemotaxis, one at 10% serum and a second one at about 50%. This is a highly reproducible curve, even though the second point of inflection does not appear to be great.

So my first question would be whether or not there are other inhibitors in Dr. Mannick's serum which might correspond to these findings.

(slide) Utilizing the concept that there were two inhibitors, we looked at G-200 Sephadex chromatography and found that there are again two inhibitors of chemotaxis. Data are confirmed using sucrose density gradients, as well as isoelectric focusing. These inhibitors are about 360,000 and 120,000 molecular weight.

More recently, we found smaller inhibitors, and it leads to my basic question. I wonder if Dr. Mannick could comment upon the nature of these multiple inhibitors, and whether they are all part of a common, or similar, immunoregulatory system.

Dr. Donald L. Morton (Los Angeles, California): Dr. Jack Roth and I have done some studies with conclusions somewhat similar to those of Dr. McLoughlin and his colleagues.

We looked at the effect of surgical trauma on immunosuppression in patients with cancer, but our data were organized in a slightly different way. We compared patients with minor trauma, such as that from regional lymphadenectomy, with patients whose operative procedures invaded the thoracic or abdominal cavities. We found no correlation with the length of operation but did find that, if the abdominal or thoracic cavities were entered, the patients were more immunosuppressed. Also, if tumor was completely resected, immunosuppressive factors disappeared even in patients who were anergic preoperatively and who had major surgical trauma.

In our series there was a correlation between blood transfusion and degree and duration of immunosuppression. The most immunosuppressed patients were those undergoing cardiopulmonary bypass.

The duration of immunosuppression in our series was similar. One patient was immunosuppressed for six weeks, but usually the patient's immunocompetence returned in seven to ten days.

Finally, I would like to ask Dr. Mannick if there was a difference in the degree of immunosuppression between patients who had cardiopulmonary bypass and those who had major aortic resections.

Dr. Stanley M. Levenson (Bronx, New York): I wonder if Dr. Mannick can tell us something about the specific amino acid composition of the active fraction or fractions. I am interested in that particularly because in the late '40's and early '50's my colleagues and I described an amino conjugate fraction which appeared in the serum of previously healthy animals and previously healthy men who were injured. The concentration of this fraction correlated with the severity of the injury. It is a dialyzable compound, or group of compounds, and increased remarkably in patients with renal dysfunction.

I was wondering whether there may be some similarity between the active fraction or fractions Dr. Mannick and his colleagues have isolated and the amino conjugate fraction we worked with in terms

of amino acid composition. I would also like to ask Dr. Mannick if he has looked at patients with renal dysfunction following injury, to see whether there is a still higher increase in the fraction or fractions he is looking at. If so, this may be one of the reasons why a patient with renal failure is particularly susceptible to infection.

DR. JOHN A. MANNICK (Closing discussion): In reply to Dr. Clowes, I do believe that we are now finding some functions for that myriad of polypeptide molecules that circulate around in everyone's serum, whose function has heretofore been unknown, and I suppose that they represent a few words in the biochemical language that cells use to communicate with one another, and that language has by no means been translated yet.

I don't know what relationship the factors that Dr. Clowes has been working with have to the one we have been talking about. I am not sure whether our factor has any metabolic effect on lymphocytes that is other than transient, simply because cells that do not rosette very well from traumatized patients in our laboratory upon washing multiple times will then rosette quite actively, and the wash material does contain the sort of molecule we have been talking about.

In answer to Dr. Meakins, I think that his idea is an intriguing one: namely, that the immunoregulatory system may have some features similar to the complement system: for example, breakdown products of one activity may subsume other activities, and we may be talking about pieces of molecules that once did something else, and now affect a different cell type. I think that is perfectly possible, but I don't know anything else to say about it at this time, other than to admit the possibility.

In reply to Dr. Morton, cardiopulmonary bypass patients and aneurysm resection patients, which were the two groups we were looking at, really behaved the same in terms of percentage that developed anergy and the percentage that had suppressive serum. So in this instance, surprisingly enough, cardiopulmonary bypass did not seem to be any different than aneurysm resection.

In answer to Dr. Levenson, I have been intrigued by that early paper of his, and he may be right. He may, in fact, have identified this material. I just don't know whether they are similar or not. The amino acid composition of our material I don't think I can give him with any confidence. We do have a sample of this material in the hands of the Molecular Biology Institute at Hoffman-La Roche, and we hope to have some information about its true nature in a few weeks.

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Correlation Between Anergy and a Circulating Immunosuppressive Factor Following Major Surgical Trauma

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In order to clarify the relationship between anergy and immunosuppressive activity in the serum, we studied 46 previously well patients before and at three, five, seven and 28 days after surgery. Delayed hypersensitivity was measured by skin testing with four common recall antigens, and serum immunosuppressive activity was determined by the ability of the patient's serum in 10% concentration to suppress by 50% or more the phytohemagglutinin (PHA) stimulation of normal human lymphocytes as compared to pooled normal serum. Prior to surgery, all patients manifested delayed hypersensitivity to one or more antigens, and no patient had immunosuppressive serum. Fifteen patients underwent minor surgery under general anesthesia and did not develop anergy or immunosuppressive serum. Thirty-one patients underwent major cardiovascular surgery. Thirteen of these patients became anergic by day 3 after operation, and 11 of the 13 developed immunosuppressive serum. Eighteen patients maintained delayed hypersensitivity after major surgery, and only three developed immunosuppressive serum. The correlation between anergy and immunosuppressive serum was highly significant ($p < 0.001$). There was a significant difference in the degree of suppressive activity in the serum of the anergic and reactive patient groups for each postoperative day studied until day 28, when there was recovery of delayed hypersensitivity and lack of immunosuppressive serum. The occurrence of postoperative anergy and immunosuppressive serum was not related to the patient's age, sex, number of perioperative blood transfusions or duration of anesthesia but was associated with an increase in postoperative infectious complications ($p < 0.05$) and in postoperative days in the hospital ($p < 0.01$). Pooled immunosuppressive serum from anergic patients was fractionated by ion exchange chromatography, gel filtration and preparative high voltage electrophoresis. The majority of the immunosuppressive activity could be accounted for by an electrophoretically homogenous polypeptide-containing fraction not identified in the serum of patients undergoing minor surgery or in normal individuals. We conclude that anergy occurring after major operative trauma is associated with the appearance of a circulating immunosuppressive molecular species and that these events are in turn associated with increased patient morbidity and increased length of hospitalization.

ANERGY IS FOUND in surgical patients with nutritional deprivation or advanced malignancy and is associated with an increased incidence of sepsis and mortality.[1-6,8] Since restoration of delayed hypersensitivity responsiveness has been reported in such patients following parenteral hyperalimentation,[3,9] this suggests an underlying mechanism for the anergy observed in these depleted individuals. On the other hand, there are conflicting reports as to whether or not major surgical trauma in nondepleted patients is followed by anergy.[5,10] Moreover, if anergy does occur under these circumstances, it is not clear what the mechanism is and what effect, if any, the anergic state has on patient morbidity and mortality. A good deal of recent investigative work has focused on defects in polymorphonuclear leukocyte function detected in anergic surgical patients.[1] While polymorphonuclear leukocytes clearly play an important role in the defense against bacterial infection in man, they have not been shown to be obligatory participants in delayed hypersensitivity responses.[11] These responses are mediated by specifically sensitized T lymphocytes, which in turn elicit the nonspecific cooperation of macrophages.

We have recently reported that major operative trauma is often followed by the appearance in the serum of a circulating factor or factors suppressive of T-lymphocyte activation.[2] We therefore undertook the present investigation in a group of well-nourished

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